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Applicant(s):	Rong Xiang et al.	
Application No.	10/090,238	
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For:	DNA VACCINES ENCODING CEA AND A CD40 LIGAND AND METHODS OF USE THEREOF	
Examiner:	Bao Qun Li	Attorney Docket No. TSRI 830.0

DECLARATION UNDER RULE 132

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Sir:

WE, RONG XIANG and RALPH A. REISFELD, declare:

1. That we are co-inventors of the invention disclosed and claimed in the above-identified application;

2. That we are aware that claims 1, 8-11, 16-18 and 36-42 continue to be rejected as unpatentable;

3. That the rejections of these claims rely, in whole or in part, on the teachings of Xiang *et al.*, *J. Immunol.* 2000, vol. 167, pp. 4560-4565 ("Xiang *et al.*"), which was published on October 15, 2001;

4. That we are co-authors of the Xiang *et al.* publication along with F. James Primus, J. Michael Ruehlmann, Andrew G. Niethammer, Steve Silletti, Holger N. Lode, Carrie S. Dolman, and Stephen D. Gillies.

5. That prior to October 15, 2001, in the United States of America, we, Rong Xiang and Ralph A. Reisfeld, had conceived, prepared and successfully tested a DNA vaccine effective for eliciting an immune response against cells that present a

carcinoembryonic antigen (CEA) comprising a plasmid DNA operably encoding a CEA; and a plasmid DNA operably encoding a CD40 ligand, together with a pharmaceutically acceptable carrier;

6. That Exhibit A, attached hereto, is a true copy of a 31-page manuscript, bearing a true date prior to October 15, 2001, but with the date obliterated therefrom, that was the manuscript from which Xiang *et al.* was published;

7. That the manuscript reproduced in Exhibit A describes a vaccine containing a plasmid DNA operably encoding CD40 ligand trimer (CD40LT) and human carcinoembryonic antigen (CEA) transfected into attenuated *S. typhimurium* bacteria, suspended in phosphate buffered saline (PBS), a pharmaceutically acceptable carrier; and

8. That the other authors listed on the Xiang *et al.* publication and on the manuscript reproduced in Exhibit A did not contribute to the invention claimed in the above-identified application.

9. That F. James Primus was listed as a co-author because he provided a mouse model used in our experiment;

10. That J. Michael Ruehlmann was a post-doctoral student working under our direct supervision;

11. That Andreas G. Niethammer was a post-doctoral student working under our direct supervision;

12. That Steve Silletti was a post-doctoral student assisting Rong Xiang;

13. That Holger N. Lode was an assistant professor assisting Rong Xiang;

14. That Carrie S. Dolman was a technician in our laboratory working under the direct supervision of Rong Xiang; and

15. That Stephen D. Gillies was a consultant to us regarding vaccine construction.

We declare further that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

La Jolla, California

Dated 9/3/2004

Rong Xiang

Dated 9/3/2004

Ralph A. Reisfeld

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MANUSCRIPT

A Dual-Function DNA Vaccine Encoding CEA and CD40 Ligand Trimer Induces T Cell-Mediated Protective Immunity against Colon Cancer in CEA-Transgenic Mice

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KEY WORDS: DNA Vaccine, Colon Cancer, CEA, CD40LT

EXHIBIT A

Running Title: Dual Function DNA Vaccine for Colon Cancer

ABSTRACT

A carcinoembryonic antigen (CEA)-based DNA vaccine encoding both CEA and CD40 ligand trimer (CD40LT) achieved effective tumor-protective immunity against murine colon carcinoma in CEA-transgenic mice by activating both naïve T cells and dendritic cells. Peripheral T cell tolerance to CEA was broken in a prophylactic model by this novel, dual-function DNA vaccine whose efficacy was further enhanced by boosts with a recombinant antibody-IL2 fusion protein (huKS1/4-IL2). These conclusions are supported by four lines of evidence. First, a lethal challenge of MC38-CEA-KSA murine colon carcinoma cells was for the first time completely rejected in 100% of experimental animals treated by oral gavage of this DNA vaccine carried by attenuated Salmonella typhimurium, followed by five boosts with huKS1/4-IL2. Second, specific activation of dendritic cells was indicated by their marked upregulation in expression of costimulatory molecules B7.1 (CD80), B7.2 (CD86) and ICAM-1. Third, a decisive increase over controls was observed in both MHC class I antigen-restricted cytotoxicity of CTLs from successfully vaccinated mice and in secretion of pro-inflammatory cytokines IFN-γ and IL12. Fourth, activation of CTLs was augmented as indicated by upregulation of activity markers LFA-1, CD25, CD28 and CD69. Taken together, these results suggest that a dual-function DNA vaccine encoding CEA and CD40LT, combined with tumor-targeted IL2, may be a promising strategy for the rational development of DNA-based cancer vaccines for future clinical applications.

INTRODUCTION

One of the major obstacles for achieving a tumor-specific immune response is to overcome peripheral T cell tolerance against tumor self-antigens and induce cytotoxic T cells (CTLs) which effectively eradicate disseminated tumor metastases and subsequently maintain a long-lasting immunological memory preventing tumor recurrence (1-4). Human carcinoembryonic antigen (CEA) is an oncofetal membrane antigen which provides a relevant tumor self-antigen target for the development of DNA vaccines for immunotherapy (5-7). A useful animal model for CEA-based vaccines was provided by the establishment of a mouse line that carries the genomic DNA transgene for human CEA (8 ,9) and expresses CEA in a tissue-specific manner similar to humans. Following in vivo priming with CEA-transfected fibroblasts, anti-CEA $CD8^+$ T cells could be elicited in these transgenic mice which were tolerant to CEA in the $CD4^+$ T cell compartment (10). Studies in humans indicated that $CD8^+$ CTLs specific for CEA were not negatively selected, similar to findings obtained with transgenic mice (11, 12).

A large body of literature describes the biological roles of CD40 ligand (CD40L), particularly its interaction with CD40 expressed on antigen presenting cells during costimulation of T cell activation (13-15). CD40L, a type II membrane protein of 35 kDa and a member of the tumor necrosis factor (TNF) gene family, is expressed on T cells upon antigen recognition (13). Members of the TNF family are biologically most active when expressed as homotrimers. CD40L is no exception in this regard and consequently was expressed as a homotrimer (CD40LT) by modification of a 33 amino acid leucine zipper motif fused to the N-terminus of the entire extracellular domain of this ligand (16). CD40LT DNA was found to enhance cellular immune responses such as induction of IFN-γ and cytolytic T cell activity when mice were vaccinated with DNA encoding the highly immunogenic model antigen β-galactosidase (17).

CD40L is critically involved in the activation of T cells necessary to induce an effective protective immunity against tumor self-antigens. Once MHC class I antigen:peptide complexes are taken up by dendritic cells (DCs) and presented to naïve T cells, the first antigen signal is delivered via T cell receptors (TCR), followed by upregulation of CD40L. On the T cell surface, CD40L then induces costimulatory activity on DCs via CD40-CD40L interactions. Thus primed, these APCs now express costimulatory molecules B7.1 (CD80) and B7.2 (CD86) which send a second costimulatory signal to T cells via interaction with CD28, an event required for full activation of T cells to concurrently produce pro-inflammatory cytokines IFN-γ and IL12 and to perform effector functions (18). The role of IL2 targeted to the tumor microenvironment by a recombinant antibody-IL2 fusion protein (huKS1/4-IL2) is to boost anti-tumor T cell responses either by acting as a second costimulatory signal in the activation of CTL (19) or by further activating pre-activated DCs expressing IL2 receptors (20, 21).

An effective means of enhancing efficacy of DNA vaccines is to grow the plasmid encoding DNA in a non-replicating strain of *Samonella typhimurium* which can then be applied as an oral vaccine. The live, attenuated bacteria transport the DNA through the gastrointestinal tract and then through the M cells which cover the Peyer's patches of the gut. From there the attenuated bacteria enter APCs such as dendritic cells and macrophages, where they die, because of their mutation, liberating multiple copies of the DNA inside the phagocytes (22).

Indeed, attenuated bacteria are believed to provide a "danger signal" (23, 24) and stimulate the innate immune system, producing pro-inflammatory cytokines like IL-12 and mediators such as nitric oxide that enhance antigen presentation and promote T_H1-type cellular immune responses associated with the eradication of tumors. In fact, attenuated *S. typhimurium* was found to be an effective carrier for an autologous oral DNA vaccine that protected against mur-

ine melanoma (25). A recombinant *Listeria monocytogenes* vaccine proved to be highly effective in mediating regression of primary murine melanoma and their established lung metastases (26). *L. monocytogenes* produces a strong cellular immune response since unlike most other intracellular bacteria it escapes into the cytoplasm by disrupting the phagosomal membrane thus allowing any protein it secretes to target both MHC class I and class II pathways of the infected cell for antigen presentation (27).

We previously achieved partial tumor-protection against a lethal challenge of MC38 murine colon carcinoma cells, stably transduced with CEA and KSA, a human pan-epithelial cell adhesion molecule. Vaccinations were accomplished by oral gavage with a CEA-based DNA vaccine carried by attenuated *Salmonella typhimurium* which induced MHC class I antigen-restricted $CD8^+$ T cell responses, resulting in rejection of subcutaneous tumors. However, this occurred in only some of the experimental mice transgenic for CEA, even when boosted with a recombinant antibody-IL2 fusion protein that targeted IL2 to the tumor microenvironment (28).

Here, we extend these findings by achieving for the first time a $CD8^+$ T cell-mediated tumor-protective immune response against CEA self-antigen effective in 100% of experimental CEA-transgenic mice which completely rejected a lethal tumor cell challenge. This was accomplished with a unique, dual function DNA vaccine encoding CEA and CD40LT, activating both DCs and naïve T lymphocytes, aided by boosts with huKS1/4-IL2 fusion protein.

MATERIALS AND METHODS

CEA Transgenic Mice: C57Bl/6J CEA-transgenic breeder mice were generated by using a 32.6 Kb AatII restriction fragment containing the entire human CEA genomic region and flanking sequences isolated from a genomic cosmid clone. A mouse cell line [C57Bl/6J-TgN (CEAGe) 18; FJP] was established (9) and CEA transgenic mice were bred at The Scripps Re-

search Institute's animal care facility. Mice were used between 6 and 8 weeks of age. All animal experiments were performed according to National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Tumor Cell Lines and Bacterial Strains: The chemically induced murine colon adenocarcinoma cell line, MC38 was stably transfected with both, CEA (C15-4.3 clone) and the epithelial cell adhesion molecule Ep-CAM/KSA (9, 29). The attenuated Salmonella typhimurium AroA$^-$ Strain SL 7207 was kindly provided by Dr. B.A.D. Stocker (Stanford University, Stanford, CA). Chemically competent *E. coli* were purchased from Invitrogen (Carlsbad, CA) and routinely grown at 37° C in LB broth or on agar plates (VWR), supplemented when necessary with 75 μg/ml ampicillin.

Construction of Expression Plasmids: We generated several distinct forms of expression plasmids to target CD40LT and CEA molecules to DCs or T cells, respectively. The plasmid used for immunization was pcDNA3.1/zeo(+) (Invitrogen). The pER-CEA control plasmid targeted to and retained in the ER, and the pW-CEA plasmid targeted to the cell surface, were described previously (28). The plasmid encoding the CD40LT gene (pCD40LT) contained a modified 33 amino acid leucine zipper motif in order to facilitate the formation of trimeric CD40L that was fused to the C-terminus of the IL7 leader sequence to direct protein expression to the cell surface or induce its secretion outside the cells (16). Detection of CD40LT by Western blotting was facilitated by incorporating a short antigenic sequence, Flag, detectable by specific monoclonal antibodies. The plasmid pCD40LT-CEA contains the entire CEA extracellular domain, fused to the C-terminus of murine CD40L, thus generating a dual-function chimeric construct.

Oral Immunization, Tumor Cell Challenge and Antibody-IL2 Fusion Protein Boosts: CEA-transgenic C57BL/6J mice were divided into seven experimental groups (n=8). Mice were immunized three times at two-week intervals by oral gavage with 100 µl PBS containing 1 x 10^8 attenuated *S. typhimurium* harboring either empty vector (pcDNA3.1), individual expression vectors pER-CEA, pW-CEA, pCD40LT, pCD40LT-CEA, or the latter followed by boosts with huKS1/4-IL2. Control experiments included PBS, antibody-IL2 fusion protein boosts without immunization by DNA vaccine and a group of mice immunized only with irradiated MC38 cells. All mice were challenged subcutaneously in the right flank with a lethal dose of 2.5x10^5 MC38-CEA-KSA cells 2 weeks after the last immunization. Mice were examined daily until the tumor became palpable after which its diameter was measured in two dimensions with a microcaliper every other day.

Construction of the huKS1/4-IL2 fusion protein has been described previously (29). C57BL/6J mice transgenic for CEA that were immunized by oral gavage with attenuated *S. typhimurium* described as above, received 5 µg boosts of huKS1/4-IL2 fusion protein for five consecutive days starting one day after tumor cell challenge.

Cytotoxicity Assay: Cytotoxicity was measured by a standard ^{51}Cr-release assay (21). Splenocytes isolated from CEA-transgenic mice, one week after tumor cell challenge, were subsequently cultured for 3 d at 37°C in complete T-STIM culture medium (Beckton Dickinson, Bedford, MA). MC38-CEA-KSA target cells (3 x 10^6), labeled with 0.5 mCi of ^{51}Cr were incubated with effector cells at various E:T ratios at 37°C for 4 h.

Transfection and Immunoblot Assessment of Protein Expression: Lipofectamine was used for transient transfection of COS-7 cells according to the manufacturer's instructions (Invitrogen), seeding COS-7 cells at 2.5x10^5 cells per well in a six-well plate and adding 24 h later, 1

μg of DNA with 5 μl lipofectamine in serum-free medium. Immunoblots were done with equal quantities of protein (15 μg/lane), separated by SDS-PAGE under reducing and non-reducing conditions alongside a control lysate and electroblotted onto a nitrocellulose membrane as described previously (21). After staining with mouse anti-human CEA mAb (ICN, Aurora, OH) or anti-FLAG M2 mAb (Sigma, St. Louis, MO), followed by anti-mouse IgG-HRP, the blot was developed with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and XOMAT-5 film (Eastman Kodak Company, Rochester, NY).

Flow Cytometry Analysis: Activation markers of T cells and expression of costimulatory molecules on CD11c and MHC class II antigen-positive DCs were determined by two-color flow cytometry analysis with a Becton Dickinson FACScan. T cell activation was determined by staining of freshly isolated splenocytes from successfully vaccinated mice with anti-CD8 FITC (53-6.7), in combination with PE-conjugated anti-CD25 (H129.19), LFA-1(2D7), CD28 (37.51) and CD69 (H1.2F3) antibodies. Activation of costimulatory molecules on APCs was measured with FITC labeled anti-CD11c (HL-3), in combination with PE-conjugated anti-B7.1 (16-10A1), B7.2 (GL1) or ICAM-1, and biotinylated anti-IA^b (KH74) antibodies followed by streptavidin-allophycocyanin. All cytometric flow experiments were performed in the presence of 0.1 μg/ml propidium iodide to exclude dead cells. All reagents were obtained from Pharmingen (La Jolla, CA).

Cytokine Induction Assay: Splenocytes were harvested from all experimental groups of mice one week after s.c. lethal tumor cell challenge with $2.5x10^5$ MC38-CEA-KSA cells. Lymphocytes were isolated on Ficoll-Hypaque (BioWhittaker, Walkersville, MD) and cultured 24 h in complete T cell medium with $1x10^5$ irradiated (15,000 rad) MC38-CEA-KSA cells. Supernatants were collected and stored at -70°C until use. Cytokines were analyzed for either IFN-γ or

IL12 with commercially available cytokine detection kits using a solid-phase sandwich ELISA (R&D Systems, Minneapolis, MN).

RESULTS

Protein Expression of CEA and CD40LT: We analyzed protein expression of plasmids: pCD40LT, pCD40LT-CEA and pW-CEA by transfection into COS-7 cells. Western blotting indicated that all constructs produced proteins of the expected molecular mass (~35 kDa, ~215 kDa and 180 kDa, respectively) as shown by SDS/PAGE analyses of lysates from transfected cells, analyzed under reducing conditions (Figs. 1A & B). A plasmid encoding pCD40LT expressed proteins in the cell lysate indicative of monomeric, dimeric and trimeric CD40L, under non-reducing conditions (Fig. 1B). CD40L protein was also detected in supernatants of transfected cells under reducing conditions (data not shown).

Induction of Tumor Protective Immunity by a Dual-Function Chimeric Molecule Encoding both CD40LT and CEA Molecules: A number of experiments performed, including several controls, indicated that the dual-function DNA vaccine targets CD40LT and CEA to DCs and T cells, respectively. Thus, C57Bl/6J mice transgenic for CEA were immunized on days 0 and 7 each by s.c. injections of $2.5x10^5$ irradiated (15,000 rad) MC38 murine colon carcinoma cells. Challenge of these controls 2 weeks later with a lethal s.c. dose of MC38-CEA-KSA cells, resulted in rapidly developing tumors in all mice indicating that MC38-CEA-KSA cells were not immunogenic per se (Fig. 2A). This was also found to be the case in three other key control experiments: Mice (n=6) vaccinated three times at 2 week intervals by oral gavage with 1×10^8 attenuated *S. typhimurium* carrying either the empty vector, the pER-CEA plasmid exclusively targeted to and retained in the ER, or the pCD40LT construct alone, all uniformly failed to elicit a protective immune response against a lethal s.c. tumor cell challenge and revealed rapid and uni-

form tumor growth (Figs. 2B, C, & D). In contrast, a group of mice treated by the same vaccination protocol, but receiving the DNA vaccine containing the pW-CEA vector, revealed a substantial decrease in tumor volume, with 3 of 8 animals completely rejecting the tumor cell challenge (Fig. 2F). In mice vaccinated with pCD40LT-CEA, 4 of 8 animals completely rejected the tumor cell challenge. In this case, the remaining mice showed a dramatic suppression of tumor growth when compared to controls ($P < 0.001$) (Fig. 2G).

Vaccination Efficacy is Amplified by Boosts with Antibody-IL2 Fusion Protein: Boosts with small, non-curative doses of huKS1/4-IL2 fusion protein targeted to the tumor microenvironment markedly increased the efficacy of the DNA vaccine. In fact, vaccination of CEA-transgenic mice by the same protocol described for the pCD40LT-CEA group, followed by i.v. injections of 5μg huKS1/4-IL2 one day after tumor cell challenge for five consecutive days, resulted in the complete rejection of the tumor cell challenge in 8/8 experimental animals (Fig. 2H). An important control experiment indicated that the injection of 5 x 5 μg of huKS1/4-IL2 fusion protein per se had essentially no effect on tumor growth, when administered to naïve mice that only received the tumor challenge without prior immunization by the DNA vaccine (Fig. 2E). The IL-2 fusion protein boost was specific since boosting with a non-specific fusion protein hu14.18-IL2 directed against ganglioside GD2 not expressed by M38 colon carcinoma cells, was quite ineffective (data not shown).

Antigen-Specific CTL Responses are Increased by the pCD40LT-CEA Dual Function Vaccine: The application of the pCD40LT-CEA vaccine induced strong cytotoxic $CD8^+$ T cell priming, either with or without huKS1/4 –IL2 fusion protein boosts, as demonstrated in CEA-transgenic mice immunized with each of the individual plasmids (Fig.3). CTLs of mice that received vaccinations with pCD40LT-CEA plus boosts with the antibody-IL2 fusion protein

proved to be most effective, inducing up to 70% lysis as compared to 45% lysis by such cells obtained from mice immunized with the same vaccine but without the fusion protein boost (Fig. 3A). In contrast, only background lysis was observed with splenocytes obtained from control animals. Tumor cell lysis was specific since the use of non-specific B16 melanoma cells lacking CEA expression as targets resulted in a complete lack of cytolysis (data not shown). Importantly, the data depicted in Figure 3B clearly demonstrate that the cytolytic response elicited by splenocytes from mice immunized against MC38-CEA-KSA tumor target cells was MHC class I antigen-restricted, since the presence of 50 μg/ml antibodies directed against H2-K^b/H2-D^b MHC class I antigens completely inhibited cytotoxic activities. This inhibitory effect was specific since the presence of non-specific anti-H-$2k^d$ and H-$2D^d$ antibodies did not inhibit cytolysis (data not shown).

Upregulation of CTL Activity Markers by the Dual-Function DNA Vaccine is Enhanced by Boosts with Antibody-IL2 Fusion Protein: The interaction between CD40LT on activated T helper cells with its CD40 target on DCs is critical for achieving optimal antigen-specific T cell responses. We observed a correlation between the ability of the dual function DNA vaccine to enhance T cell-dependent immune responses and the increase in expression of T cell activation markers. This was evident from increases in expression of CD25, the high affinity IL2 receptor α chain, CD69, an early T cell activation antigen, and the lymphocyte function-associated antigen, LFA-1, important for the initial interaction between T cells and DCs via the intercellular cell adhesion molecule, ICAM-1 (Fig. 4). Importantly, these upregulated T cell activation markers also included CD28, a member of the Ig superfamily expressed on T cells which serves as the receptor for the costimulatory B7.1 and B7.2 molecules of DCs whose ligation with CD28, in turn, will costimulate growth of naïve T cells (Fig. 4). Importantly, boosts with

huKS1/4-IL2 fusion proteins 24 h after tumor cell challenge further elevated expression of these same markers by 20% to 35%.

Increased Expression of Costimulatory Molecules by Immunization with pCD40LT-CEA Molecules and Boosts by Antibody-IL2 Fusion Protein: T cell activation is critically dependent on upregulated expression of costimulatory molecules B7.1 and B7.2 on DCs to achieve optimal ligation with CD28 expressed on T cells. Equally important is the upregulation of ICAM-1 which binds the T cell integrin LFA-1. FACS analyses of splenocytes obtained from CEA-transgenic mice, successfully immunized with the DNA vaccine and boosted with antibody-IL2 fusion protein, clearly indicated that we accomplished this particular task very effectively as the expression of B7.1, B7.2 and ICAM-1 was upregulated one to two-fold over that of controls (Fig. 5). Boosts with antibody-IL2 fusion protein resulted in an additional 20% to 40% increase in expression of both costimulatory and adhesion molecules (Fig. 5). These data provide evidence that vaccination with pCD40LT-CEA molecules induce and enhance the expression of costimulatory molecules on $CD11c^+$ and MHC class II antigen-positive DCs, suggesting that the capability of these APCs for tumor specific antigen processing and presentation was significantly increased.

pCD40LT-CEA Vaccination Enhances Production of Cytokines Boosted Further by Antibody-IL2 Fusion Protein: The pCD40LT-CEA vaccine enhanced the release of pro-inflammatory cytokines, IFN-γ and IL12 from T cells, as indicated by a solid-phase sandwich ELISA measuring their production in supernatants of various splenocyte preparations 24 h after being plated in the presence of irradiated (15,000 rad) MC38-CEA-KSA tumor cells. Only background levels of IFN-γ and IL12 were detected when analyzing supernatants of splenocytes obtained from PBS treated CEA-transgenic control mice after challenge with MC38-CEA-KSA

cells. However, if mice received the pCD40LT-CEA DNA vaccine, the production of IFN-γ and IL12 increased by 75% and 50%, respectively, over those levels observed in mice vaccinated with either pCD40LT or pW-CEA alone (Fig. 6). Production of IFN-γ was further augmented by 25% after boosts with huKS1/4-IL2 fusion protein, while that of IL12 increased 3-fold over control values and 100% over that observed after vaccination, but without the huKS1/4-IL2 boost (Fig. 6). These data support the contention that DNA immunization coupled with boosts of antibody-IL2 fusion protein decisively increased T cell activation in secondary lymphoid tissues.

DISCUSSION

The major objective of this study was achieved by breaking peripheral T cell tolerance against CEA, a human tumor self-antigen with a dual-function oral DNA vaccine encoding both CEA and CD40LT in CEA-transgenic mice. Importantly, a $CD8^+$ T cell-mediated rejection of a lethal challenge of murine colon carcinoma cells occurred that was completely effective in 100% of experimental mice in a prophylactic setting. Although we, as well as other investigators (28, 30-32), previously reported that tumor-protective immunity was achieved with a CEA-based DNA vaccine in CEA-transgenic mice, this treatment was never completely effective in all experimental animals. We attribute the completely successful tumor-protective immunity achieved in all CEA-transgenic mice in this study to the combined action of the unique dual-function DNA vaccine and the huKS1/4-IL2 fusion protein, which accomplished the concurrent activation of both antigen-presenting DCs and naïve T cells. Possible mechanisms of action were suggested by the upregulated expression of several receptor/ligand pairs known to critically impact effective activation of T cells following their interaction with DCs which present them with MHC:peptide complexes. These included CD40/CD40LT, LFA-1/ICAM-1, CD28/B7.1 and B7.2 and CD25/IL2 as well as the increased secretion of pro-inflammatory cytokines IFN-γ and

IL12. Several lines of evidence provided insights into the priming of $CD8^+$ T cells *in vivo* following immunization with the pCD40LT-CEA dual-function DNA vaccine and challenge with colon carcinoma cells. First, a marked activation of T cells and $CD11c^+$ dendritic-like cells was indicated by the decisive upregulation in expression of T cell integrins LFA-1 and ICAM-1 which are known to synergize in the binding of lymphocytes to APCs (33). In fact, the transient binding of naïve T cells to APCs is crucial in providing time for these cells to sample large numbers of MHC molecules on the surface of each APC for the presence of specific peptides. This mechanism would increase the chance of a naïve T cell recognizing its specific MHC:peptide ligand, followed by signaling through the TCR and induction of a conformational change in LFA-1. This, in turn, will greatly increase LFA-1's affinity for ICAM-1 and stabilize the association between the antigen-specific T cell and the APC (34, 35). Second, the marked increase in expression of CD28 on T cells as well as the costimulatory molecules B7.1 and B7.2 on DCs, following vaccination and tumor cell challenge is particularly significant since it provides the two signals required for activation of naïve T cells. One signal, indicating antigen recognition being transmitted to T cells after binding of the MHC: peptide complex to the TCR, and the other signal, ligation of CD28 with B7.1 and B7.2, initiating T cell responses and production of armed effector T cells (13, 15, 18). Third, a clear indication of T cell activation in secondary lymphoid tissues was provided by marked increases in expression of CD25, the high affinity IL2 receptor α chain and CD69, an early T cell activation antigen.

The significant elevation in the production of pro-inflammatory cytokines IFN-γ and IL12 by T cells induced by our dual-function DNA vaccine suggests that a third signal may act directly on T cells (23, 24, 36). This "danger signal", was reported to be required for T_H1 differentiation leading to clonal expansion of T cells (36). In fact, whenever T cell help is required to

generate an effective $CD8^+$ T cell response against a tumor-self antigen like CEA, triggering of DCs is necessary prior to their encounter with an antigen-specific $CD8^+$ T cell (37). This effect is mediated by ligation of CD40 on the surface of APCs (35) with CD40L expressed on activated $CD4^+$ T cells. CD40LT expressed by our DNA vaccine likely acted as a surrogate for activated $CD4^+$ T cells, leading to maturation of DCs as indicated by their decisive upregulation of B7.1 and B7.2 costimulatory molecules (38). In summary, we demonstrated that our orally administered dual-function DNA vaccine containing genes encoding for both CEA and CD40LT induced a highly efficient tumor-protective immunity against CEA self-antigen in all experimental CEA-transgenic mice. It is anticipated that this strategy may ultimately lead to an improvement in the efficacy of DNA vaccines for cancer therapy.

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REFERENCES

1. Boon, T., Cerottini, J. C., Van den Eynde, B., van der Bruggen, P., and Van Pel, A. 1994. Tumor antigens recognized by T lymphocytes. *Annu. Rev. Immunol. 12:337.*

2. Houghton, A. N. 1994. Cancer antigens: immune recognition of self and altered self. *J. Exp. Med., 180:1.*

3. Gilboa, E., Nair, S. K., and Lyerly, H. K. 1998. Immunotherapy of cancer with dendritic-cell-based vaccines. *Cancer Immunol. Immunother. 46:82.*

4. Gurunathan, S., Klinman, D. M., and Seder, R. A. 2000. DNA vaccines: immunology, application and optimization. *Annu. Rev. Immunol. 18:927.*

5. Thompson, J., and Zimmermann, W. 1988. The carcinoembryonic antigen gene family: structure, expression and evolution. *Tumor Biol. 9:63.*

6. Sharkey, R. M., Goldenberg, D. M., Goldenberg, H., Lee, R. E., Balance, C., Powlyk, D., Varga, D., and Hansen, H. J. 1990. Murine monoclonal antibodies against carcinoembryonic antigen: pharmacokinetic and targeting properties in humans. *Cancer Res. 50:2823.*

7. Kantor, J., Irvine, K., Abrams, S., Kaufmann, H., DiPietro, J., and Schlom, J. 1992. Anti-tumor activity and immune responses induced by a recombinant vaccinia-carcinoembryonic antigen (CEA) vaccine. *J. Natl. Cancer Inst., 84:1084.*

8. Schrewe, H., Thompson, J., Bona, M., Hefta, L. J. F., Maruya, A., Hassauer, M., Shively, J. E., von Kleist, S., and Zimmermann, W. 1990. Cloning of the complete gene for carcino-embryonic antigen: analysis of its promoter indicates a region conveying cell type-specific expression. *Mol. Cell. Biol. 10:2738.*

9. Clarke, P., Mann, J., Simpson, J. F., Ricard-Dickson, K. J., and Primus, F. J. 1998. Mice transgenic for human carcinoembryonic antigen as a model for immunotherapy. *Cancer Res. 58:1469.*

10. Mizobata, S., Tompkins, K., Simpson, J. F., Shyr, Y., and Primus, F. J. 2000. Induction of cytotoxic T cells and their anti-tumor activity in mice transgenic for carcinoembryonic antigen. *Cancer Immunol. Immunother. 49:285.*

11. Tsang, K. Y., Zaremba, S., Nieroda, C. A., Zhu, M. Z., Hamilton, J. M., and Schlom, J. 1995. Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. *J. Natl. Cancer Inst. 87:982.*

12. Kawashima, I., Hudson, S. J., Tsai, V., Southwood, S., Takesako, K., Appella, E., Sette, A., and Celis, E. 1998. The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. *Hum. Immunol. 59:1.*

13. Grewal, I. S., and Flavell, R. A. 1996. The role of CD40 ligand costimulation in T cell activation. *Immunol. Rev. 153:85.*

14. Toes, R. E. M., Schoenberger, S. P., Van Der Voort, E. I. H., Offringa, R., and Melief, C. J. 1998. CD40-CD40 ligand interactions and their role in cytotoxic T lymphocyte priming and anti-tumor immunity. *Semin. Immunol. 10:443.*

15. Whitmire, J. K., Flavell, R. A. Grewal, I. S., Larsen, C. P., Pearson, T. C., and Ahmed, R. 1999. CD40-CD40 ligand costimulation is required for generating anti-viral $CD4^+$ T cell responses but is dispensable for $CD8^+$ T cell responses. *J. Immunol. 163:3194.*

16. Fanslow, W. C., Srinivasan, S., Paxton, R., Gibson, M. G., Spriggs, M. K., and Armitage, R. J. 1994. Structural characteristics of CD40 ligand that determine biological function. *Semin. Immunol. 6:267.*

17. Gurunathan, S., Irvine, K. R., Wu, C. Y.,Cohen, J. I., Thomas, E., Prussin, C., Restito, N. P., and Seder, R. A. 1998. CD40 ligand/trimer DNA enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. *J. Immunol. 161:4563.*

18. Grewal, I. S., and Flavell, R. A. 1998. CD40 and CD154 in cell-mediated immunity. *Annu. Rev. Immunol. 16:111.*

19. Sprent, J. 1995. Antigen-presenting cells. Professionals and amateurs. *Curr. Biol. 95:1095.*

20. Maass., G., Schmidt, W., Berger, M., Schilcher, F., Koszik, F., Schneeberger, A., Stingl, G., Birnstiel, M. L., and Schweighoffer, T. 1995. Priming of tumor-specific T cells in the draining lymph nodes after immunization with interleukin 2-secreting tumor cells: three consecutive stages may be required for successful tumor vaccination. *Proc. Natl. Acad. Sci. USA 95:5540.*

21. Xiang, R., Lode, H. N., Dolman, C. S., Dreier, T., Varki, N. M., Qian, X., Lo, K. M., Lan, Y., Super, M., Gillies, S. D., and Reisfeld, R. A. 1997. Elimination of established murine colon carcinoma metastases by antibody-interleukin-2 fusion protein therapy. *Cancer Res. 57:4948.*

22. Darji, A., Guzman, G. A., Gerstel, B., Wachholz, P., Timmis, K. N., Wehland, T., Chakraborty, E., and Weiss, S. 1997. Oral somatic transgene vaccination using attenuated *S. typhimurium. Cell. 91:765.*

23. Matzinger, P. 1994. Tolerance, danger and the extended family. *Annu. Rev. Immunol. 12:99.*

24. Matzinger, P. 1998. An innate sense of danger. *Semin. Immunol. 10:399.*

25. Xiang, R., Lode, H. N., Ta-Hsiang, C., Ruehlmann, J. M., Dolman, C. S., Rodriguez, F., Whitton, J. L., Overwijk, W. W., Restifo, N. P., and Reisfeld, R. A. 2000. An autologous oral DNA vaccine protects against murine melanoma. *Proc. Nat. Acad. Sci. (USA) 97:5492.*

26. Pan, Z. K., Weiskirch, L. M., and Paterson, Y. 1999. Regression of established B16 F10 melanoma with a recombinant *Listerra monocytogenes* vaccine. *Cancer Res. 59:5264.*

27. Weiskirch, L. M., and Paterson, Y. 1997. *Listerra monocytogenes*: a potent vaccine vector for neoplastic and infectious disease. *Immunol. Rev. 158:159.*

28. Xiang, R., Silletti, S., Lode, H. N., Dolman, C. S., Ruehlmann, J. M., Niethammer, A. G., Pertl, U., Gillies, S. D., Primus, F. J., and Reisfeld, R. A. 2001. Protective immunity against human carcinoembryonic antigen (CEA) induced by an oral DNA vaccine in CEA-transgenic mice. *Clin. Cancer Res. 3:856s.*

29. Gillies, S. D., Lan, Y., Wesolowski, K. S., Qian, X., Reisfeld, R. A., Holden, S., Super, M., and Lo, K. M. 1998. Antibody-IL12 fusion proteins are effective in SCID mouse models of prostate and colon carcinoma metastases. *J. Immunol. 160:6195.*

30. McLaughlin, J. P., Schlom, J., Kantor, J. A., and Greiner, J. W.1996. Improved immuno-therapy of a recombinant carcinoembryonic antigen vaccinia vaccine when given in comb-ination with interleukin-2. *Cancer Res. 56:2361.*

31. Hodge, J. W., Sabzevari, H., Gómez-Yatal, A., Gritz, L., Lorenz, M. G. O., and Schlom, J. 1999. A triad of co-stimulatory molecules synergizes to amplify T cell activation. *Cancer Res. 59:5800.*

32. Kass, E., Schlom, J., Thompson, J., Guadagni, F., Graziano, P., and Greiner, J. W. 1999. Induction of protective host immunity to carcinoembryonic antigen (CEA), with a recombinant vaccinia-CEA virus. C*ancer Res.* 59:676.

33. Dustin, M. L., and Springer, T. A. 1993. The role of lymphocyte adhesion receptors in transient interactions and cell locomotion. *Annu. Rev. Immunol. 9:27.*

34. Dustin, M. L., and Springer, T. A. 1989. T cell receptor crosslinking transiently stimulates adhesiveness through LFA-1. *Nature 341:619.*

35. Hogg, N., and Landis, R. C. 1993. Adhesion molecules in cell interactions. *Curr. Opin. Immunol. 5:383.*

36. Curtsinger, J. M., Schmidt, C. S., Mondino, A., Lins, D. C., Kedl, R. M., Jenkins, M. K., and Mescher, M. F. 1999. Inflammatory cytokines provide a third signal for activation of naïve $CD4^+$ and $CD8^+$ T cells. *J. Immunol.* 162:3256.

37. Chambers, C. A. and Allison, J. P. 1999. Co-stimulatory regulation of T cell function. *Curr. Opin. Cell Biol.* 11:203.

38. Schoenberger, S. P., Toes, R. E., Van Der Voort, E. I., Offringa, R., and Melief, C. J. M. 1998. T cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393:480.

FOOTNOTES

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[3]ABBREVIATIONS: CD40LT, CD40 ligand trimer; DCs, dendritic cells; EpCAM, epithelial cell adhesion molecule; CEA, carcinoembryonic antigen.

LEGENDS TO FIGURES

Fig. 1. Detection of protein expression of plasmids by Western blotting. Cos-7 cells were transfected with pCD40LT, pCD40LT-CEA and pW-CEA plasmids and cell lysates were analyzed under reducing or non-reducing conditions. **A.** Blot was probed with anti-human CEA, under reducing conditions. **B.** Blot was probed with anti-FLAG M2 to show CD40L expression under reducing and non-reducing conditions.

Fig. 2. Growth prevention of MC38-CEA-KSA tumors by an oral DNA vaccine and boosts with huKS1/4-IL2 fusion protein. Mice in group A were immunized with irradiated MC38-CEA-KSA colon carcinoma cells on days 0 and 7. Experimental animals in groups B, C, D, F, G and H were each immunized three times by oral gavage at two-week intervals with 1 x 10^8 attenuated *S. typhimurium* harboring the individual plasmids indicated. Two weeks later, all mice were challenged s.c. with a lethal dose of 2.5 x 10^5 MC38-CEA-KSA cells. Group H was vaccinated and then boosted i.v. with five doses (5 μg each) of huKS1/4-IL2 fusion protein and naïve mice (E) were treated only with fusion protein as a control. Animals were examined daily until the tumor became palpable, after which its diameter was measured with microcalipers in two dimensions every other day. The tumor growth of each individual mouse is depicted by a solid line.

Fig. 3. Priming of CTLs by DNA vaccine and functional amplification by huKS1/4-IL2. C57Bl/6J mice transgenic for CEA were vaccinated as described in Fig. 2. Splenocytes of mice were isolated one week after tumor cell challenge, i.e., the second day after completion of boosts with fusion protein and analyzed for their cytotoxic activity in a 4 h ^{51}Cr-release assay at different E:T ratios. **A.** *T cell-mediated cytotoxicity.* MC38-CEA-KSA cells served as targets for splenocytes isolated from untreated tumor-bearing mice (□), mice treated only with fusion protein

(◇), mice immunized with plasmids pER-CEA (o), pCD40LT(Δ), pW-CEA (⊞), pCD40LT-CEA(◆) and pCD40LT-CEA plus huKS1/4-IL2 boosts(⊕). **B.** *Inhibition of MHC class I antigen-restricted lysis of tumor cells by CTLs.* Blocking of cytotoxicity was done in the presence of 50 μg/ml of anti-MHC class I antibody (H-2K^b/H-2D^b) in the same groups of mice. Each value shown represents the mean of 4 mice.

Fig. 4. Upregulated expression of T cell activation molecules. C57Bl/6J mice transgenic for CEA were immunized with the DNA vaccine and then challenged with tumor cells and boosted with huKS1/4-IL2 fusion protein, as described in Fig. 2. FACS analyses were done with splenocytes obtained from each experimental group, at the time point indicated. Two-color flow cytometry analyses were performed with single cell suspensions of splenocytes. Anti-LFA-1, CD25, CD28 and CD69 antibodies were used in PE-conjugated form in combination with FITC-conjugated anti-mouse mAb CD3e. Each value represents the mean and standard deviation for 4 mice.

Fig. 5. pCD40LT-CEA vaccination plus huKS1/4-IL2 fusion protein boosts enhanced the expression of costimulatory molecules. In the same experiment outlined in Fig. 4, two-color flow cytometric analyses were performed with single cell suspensions prepared from splenocytes obtained one day after fusion protein boosts. Splenocytes were stained with FITC labeled anti-CD11c antibody, in combination with PE conjugated anti-B7.1, B7.2 or ICAM-1 antibodies together with biotinylated anti-IA^b antibody followed by streptavidin-allophycocyanin. Shown are cell surface expressions of costimulatory molecules B7.1, B7.2 and ICAM-1. Each value represents the mean and standard deviation for 4 mice.

Fig. 6. Induction of pro-inflammatory cytokines. C57Bl/6J mice transgenic for CEA were immunized with the pCD40LT-CEA vaccine with and without boosts of huKS1/4-IL2 fusion protein as well as with the pW-CEA vaccine and controls of pCD40LT and PBS and then challenged with MC38-CEA-KSA tumor cells. Splenocytes were obtained 1 week after tumor cell challenge and plated in the presence of irradiated MC38-CEA-KSA cells. Culture supernatants were then harvested after 24 h and analyzed for release of either IFN-γ (A) or IL12 (B) by a solid-phase ELISA. Each value represents the mean and standard deviation for 4 mice.